

(11) **EP 0 633 930 B1**

(12) **EUROPEAN PATENT SPECIFICATION**

(45) Date of publication and mention  
of the grant of the patent:  
**26.04.2000 Bulletin 2000/17**

(51) Int. Cl.<sup>7</sup>: **C12N 5/08**

(21) Application number: **93908477.8**

(86) International application number:  
**PCT/US93/02639**

(22) Date of filing: **29.03.1993**

(87) International publication number:  
**WO 93/20186 (14.10.1993 Gazette 1993/25)**

(54) **IN VITRO GENERATION OF HUMAN DENDRITIC CELLS**

**IN VITRO BILDUNG VON DENDRITISCHEN ZELLEN**

**PRODUCTION (IN VITRO) DE CELLULES DENDRITIQUES HUMAINES**

(84) Designated Contracting States:  
**AT BE CH DE DK ES FR GB GR IE IT LI LU MC NL  
PT SE**

(56) References cited:  
**EP-A- 0 455 482 WO-A-91/02531  
WO-A-91/06630**

(30) Priority: **30.03.1992 EP 92400879**

(43) Date of publication of application:  
**18.01.1995 Bulletin 1995/03**

(73) Proprietor: **SCHERING CORPORATION**  
**Kenilworth New Jersey 07033 (US)**

(72) Inventors:  
• **BANCHEREAU, Jacques**  
**F-69130 Ecully (FR)**  
• **CAUX, Christophe**  
**F-69005 Lyon (FR)**

(74) Representative:  
**Ritter, Stephen David**  
**Mathys & Squire**  
**100 Grays Inn Road**  
**London WC1X 8AL (GB)**

- **EUROPEAN JOURNAL OF IMMUNOLOGY** vol. 20, no. 6, 1990, WEINHEIM DE pages 1265 - 1272 Seman M.; Boudaly S.; Roger T.; Morisset J.; Pham G. 'Autoreactive T cells in normal mice: Unrestricted recognition of self peptides on dendritic cell I-A molecules by CD4-CD8- T cell receptor alpha/beta+ T cell clones expressing V(beta)8.1 gene segments'
- **JOURNAL OF EXPERIMENTAL MEDICINE** vol. 175, no. 2, 1 February 1992, NEW YORK US pages 609 - 612 Nair S; Zhou F; Reddy R; Huang L; Rouse BT 'Soluble proteins delivered to dendritic cells via pH-sensitive liposomes induce primary cytotoxic T lymphocyte responses in vitro.'
- **IMMUNOLOGY** vol. 74, no. 3, 1991, OXFORD GB pages 399 - 406 MACATONIA S.E.; PATTERSON S.; KNIGHT S.C. 'Primary proliferative and cytotoxic T-cell responses to HIV induced in vitro by human dendritic cells'
- **EMBASE DATABASE**, Amsterdam, Ab.No. 91344512 Nishimura T. et al. 'Bispecific antibody-directed...'
- **BLOOD** vol. 75, no. 12, 1990, NEW YORK pages 2292 - 2298 CAUX C; SAELAND S; FAVRE C; DUVERT V; MANNONI P; BANCHEREAU J 'TUMOR NECROSIS FACTOR-ALPHA STRONGLY POTENTIATES INTERLEUKIN-3 AND GRANULOCYTE-MACROPHAGE COLONY-STIMULATING FACTOR-INDUCED PROLIFERATION OF HUMAN CD34-POSITIVE HEMATOPOIETIC PROGENITOR CELLS'

Note: Within nine months from the publication of the mention of the grant of the European patent, any person may give notice to the European Patent Office of opposition to the European patent granted. Notice of opposition shall be filed in a written reasoned statement. It shall not be deemed to have been filed until the opposition fee has been paid. (Art. 99(1) European Patent Convention).

**EP 0 633 930 B1**

• JOURNAL OF INVESTIGATIVE  
DERMATOLOGY vol. 97, no. 3, 1991,  
BALTIMORE US pages 524 - 528 C.MOULON  
ET AL. 'A potential role for Cd1a molecules...

• JOURNAL OF INVESTIGATIVE  
DERMATOLOGY vol. 94, no. 2, 1990,  
BALTIMORE US pages 166 - 173 TEUNISSEN  
MB; WORMMEESTER J; KRIEG SR; PETERS  
PJ; VOGELS IM; KAPSENBERG ML; BOS JD  
'Human epidermal Langerhans cells undergo  
profound morphologic and phenotypical  
changes during in vitro culture.'

---

## Description

[0001] The invention relates generally to an *in vitro* method of generating human dendritic cells, and, more specifically, to therapeutic and diagnostic uses of the generated cells.

5 [0002] Dendritic cells are a system of antigen-presenting cells that function to initiate several immune responses such as the sensitization of MHC-restricted T cells, the rejection of organ transplants, and the formation of T cell-dependent antibodies. Dendritic cells are found in many nonlymphoid tissues but can migrate *via* the afferent lymph or the blood stream to the T cell-dependent areas of lymphoid organs. They are found in the skin, where they are named Langerhans cells, and are also present in the mucosa. They represent the sentinels of the immune system within the  
10 peripheral tissues where they can acquire antigens. As these cells express CD4 and can be infected *in vitro* by HIV, they are likely to present a port of entry of HIV virus *in vivo*: e.g. Knight et al., pp. 145 in Racz, et al., editors, "Accessory Cells in HIV and Other Retroviral Infections" (Karger, Basel, 1991); Ramsauer et al., pp. 155 in Racz, et al., editors (cited above). The isolation of human dendritic cells from peripheral blood has only recently been achieved and only small numbers of cells can be generated, e.g. Freudenthal et al., *Proc. Natl. Acad. Sci.*, Vol. 87, pp. 7698 (1990). The  
15 *in vitro* generation of large numbers of human dendritic cells would present an important advantage for priming *in vitro* human naive CD4 and CD8 T cells, for screening agents that may interfere with HIV infection, for generating primary and secondary *in vivo* response of human B cells in SCID-hu mice reconstituted with human T and B cells, and for constructing a more sensitive mixed-lymphocyte reaction assay.

[0003] A major impediment to transplantation of allogeneic tissue and organs is graft rejection by the transplant  
20 recipient. The cell-mediated immune reaction of the recipient, or host, to the donor tissue plays an important role in the rejection process. The cell-mediated immune response has two important phases: (i) recognition, when host cells recognize the donor cell as foreign in the context of the major histocompatibility complex (MHC); and (ii) destruction, when the host cells respond by attacking the foreign cells. As part of the attacking process, a number of responder cells undergo proliferation and acquire cytotoxicity - that is, the ability to kill donor cells displaying the appropriate antigens.  
25 Thus, cell-mediated immunity can be described in terms of two measurable functions: proliferation, and cytotoxic activity - see Dubey et al., chapter 131 in Rose et al., Editors, "Manual of Clinical Laboratory Immunology", 3rd edition (American Society of Microbiology, Washington, D.C., 1986).

[0004] Development of cell culture techniques has led to the establishment of *in vitro* methods that mimic the *in vivo* immunization process, thus providing measures for the assessment of cell-mediated immunity *in vitro*. Of particular utility  
30 in regard to transplantation is the mixed lymphocyte response (MLR), or mixed lymphocyte culture. The MLR is a relatively simple assay, yet it exists in many variants. Typically, the assay consists of mixing responder lymphocytes in a suitable culture system with stimulator lymphocytes whose proliferation and/or transcription machinery has been disabled, e.g. by irradiation. After the cells have been cultured for several days, a number of different measurements can be made to quantify the degree of reactivity of the responder cells to the stimulator cells, e.g. uptake of tritiated thymidine, number of blast cells, number of dividing cells, cytokine production, and the like. Other variables in the assay  
35 include the source of the responder and stimulator cells, e.g. peripheral blood, spleen, lymph nodes, etc.; whether the responder cells are syngeneic, allogeneic, or xenogenic with respect to the stimulator cells; the method of disabling the stimulator cells, for example irradiation or treatment with a DNA synthesis inhibitor (e.g. mitomycin C) or the like.

[0005] A drawback of the MLR as a routine assay for cell-mediated immune reactivity is sensitivity. Frequently, it is  
40 difficult to obtain a strong effect in the MLR, whatever the particular read-out employed. It is believed that antigen-presenting cells in the stimulator population are responsible for stimulating the responder cells; however, in most tissue sources such cells are few in number and/or are of a type that stimulates inefficiently. The sensitivity, and hence the utility, of the MLR assay could be greatly enhanced by the availability of more potent stimulator cell populations. Dendritic cells could serve this function, since they are well known as potent antigen-presenting cells, e.g. Steinman, *Ann. Rev. Immunol.*, Vol. 9, pgs. 271-296 (1991). Unfortunately, it is presently very difficult to obtain them in quantities sufficient for routine MLRs, e.g. Steinman, et al., chapter 49 in Herzenberg et al., Editors, "Cellular Immunology" Vol. 2 (Blackwell Scientific Publications, Oxford, 1986).

[0006] In this regard, EP 0 455 482 discloses a substantially pure population of human cells comprising pluripotent cells that express the CD34 antigen, but lack expression of the CD38 antigen and other lineage associated antigens.  
50 However, the production of human dendritic cells is not disclosed.

[0007] Similarly, an article entitled "Tumor Necrosis Factor- $\alpha$  Strongly Potentiates Interleukin-3 and Granulocyte-macrophage Colony-Stimulating Factor-Induced Proliferation of Human CD34<sup>+</sup> Hematopoietic Progenitor Cells", by Caux, C. et al.; *Blood*, Vol. 75, No. 12, (June 15), 1990, pp. 2292-2298, discloses the potentiation by TNF- $\alpha$  of the proliferation of human CD34<sup>+</sup> cells by IL-3 and GM-CSF. However, the production and recovery of CD1a human dendritic cells is not described or suggested.  
55

Summary of the Invention

[0008] The invention is directed to a method for *in vitro* generation of human dendritic cells. The invention also includes isolated populations of human dendritic cells produced by the method of the invention and applications of the isolated cells, including an improved MLR assay that employs a pure population of dendritic cells as stimulator cells.

[0009] According to the invention there is provided a process for producing human dendritic cells comprising the steps of:

- (a) culturing human CD34<sup>+</sup> hematopoietic cells (i) with GM-CSF, (ii) with TNF- $\alpha$  and IL-3, or (iii) with GM-CSF and TNF- $\alpha$ , thereby inducing the formation of human dendritic cells from said CD34<sup>+</sup> hematopoietic cells; and
- (b) recovering said human dendritic cells from said culture.

[0010] Dendritic cells initiate immunological responses. The *in vitro* data reported herein for the dendritic cells produced according to the invention indicate that these cells are useful as laboratory tools and also may have utility in the *in vivo* treatment of various diseases by adoptive immunotherapy, including cancer and viral infections.

Brief Description of the Figures

[0011] The invention will now be described in more detail with particular reference to the accompanying drawings of which:

Figure 1 illustrates flow cytometry data showing the partial co-expression of CD14 by CD1a<sup>+</sup> cells.

Figure 2 illustrates data concerning the growth kinetics of CD1a<sup>+</sup> cells under various medium conditions.

Figure 3 illustrates data of CD4<sup>+</sup> cells proliferation after stimulation by dendritic cells.

Figure 4 illustrates data showing the effect of CD1a<sup>+</sup> cells in stimulation of allogeneic CD4<sup>+</sup> cell proliferation.

Figure 5 illustrates data of CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation after stimulation by dendritic cells.

DETAILED DESCRIPTION OF THE INVENTION

[0012] An important aspect of the invention is the generation of dendritic cells from CD34<sup>+</sup> hematopoietic cells. CD34<sup>+</sup> hematopoietic progenitor cells can be obtained from a variety of tissue sources, *e.g.* bone marrow, but are preferably obtained from umbilical cord blood samples as follows: Light-density mononuclear cells from the samples are isolated by Ficoll-Hypaque gradient separation ( $d = 1.077 \text{ g/mL}$ ) and are depleted of adherent cells, *e.g.* by overnight incubation at 37°C in RPMI 1640 medium supplemented with 1% w/v tissue-culture grade bovine serum albumin. Preferably, cells bearing CD34 antigen are isolated from non-adherent mononuclear fractions through positive selection by indirect immune panning using anti-CD34 monoclonal antibody, *e.g.* Imu-133.3 available from Immunotech (Marseille, France), anti-My 10 available from Becton Dickinson (Mountain View, California), or the like. Panning flasks are prepared as follows: sheep Fab antimouse IgG at a concentration of 25  $\mu\text{g/mL}$  in Tris buffer (0.05 mol/L, pH 9.4) is distributed (10 mL) in 75-cm<sup>2</sup> tissue culture flasks for overnight coating at 4°C. Separately, the light-density mononuclear cells (depleted of adherent cells as discussed above) are incubated one hour at 4°C with 5  $\mu\text{g/mL}$  anti-CD34 antibody at 10<sup>7</sup> cells/mL in RPMI 1640 supplemented with 2% heat-inactivated pooled human AB serum (HABS). Afterwards, cells are washed in cold medium containing 2% HABS, and 10 mL containing about  $5 \times 10^7$  cells are distributed to the flasks previously coated with sheep antimouse IgG, as described above. Following a two-hour incubation at 4°C, non-adherent cells in suspension (*i.e.* the CD34-depleted fraction) are harvested by gentle pipetting and rinsing several times with medium. The adherent "panned" cells (*i.e.* the CD34-rich fraction) are then recovered by vigorous pipetting.

[0013] The dendritic cells are obtained from the CD34<sup>+</sup> cells by culturing these in medium containing GM-CSF or TNF- $\alpha$  and IL-3. Preferably, the dendritic cells are obtained from the CD34<sup>+</sup> cells by culturing these in medium containing TNF- $\alpha$  as well as GM-CSF. TNF- $\alpha$ , GM-CSF, and IL-3 suitable for use in the invention are commercially available, *e.g.* from Genzyme Corp. (Cambridge, MA), or can be produced by recombinant expression systems: *e.g.* as taught by Clark et al. in U.S. patent 4,959,455 (IL-3); Clark et al. in PCT application No. EP85/00326 (publ. no. WO86/00639) (GM-CSF); and Mark et al. in U.S. patent 4,677,063 (TNF- $\alpha$ ). Preferably, IL-3 and GM-CSF are used at saturating concentration; that is, they are used at a concentration at which all the IL-3 and GM-CSF receptors on the CD34<sup>+</sup> cells are occupied by biologically active IL-3 and GM-CSF molecules. Of course, the actual concentration may depend on the quality of IL-3 and GM-CSF used. Preferably, human IL-3 having a specific activity of at least  $5 \times 10^6 \text{ U/mg}$  is employed,

wherein a unit of activity corresponds to the half-maximum proliferative activity as determined by  $^3\text{H}$ -thymidine uptake by human bone marrow cells in liquid cultures. In the culture systems described below, saturating concentration was 10 ng/mL (or 50 U/mL). Preferably, human GM-CSF having a specific activity of at least  $2 \times 10^6$  U/mg is employed, wherein a unit of activity is as defined for IL-3 above. In the culture systems described below, saturating concentration was 100 ng/mL (or 200 U/mL). Preferably, TNF- $\alpha$  is used at a concentration in the range of 2 to 3 ng/mL or 40-60 U/mL, most preferably at a concentration of about 2.5 ng/mL or 50 U/mL. Units of TNF- $\alpha$  are defined by Carswell et al., *Proc. Natl. Acad. Sci.*, Vol. 72, pg. 3666 (1975), and by Aggarwal et al., *J. Biol. Chem.*, Vol. 260, pg. 2345 (1985). The cells can be co-cultured in standard tissue culture medium with standard additives, such as RPMI 1640 supplemented with 10% (v/v) heat inactivated fetal bovine serum, 10 mM Hepes, 2 mM L-glutamine,  $5 \times 10^{-5}$  M 2-mercaptoethanol, penicillin (100 U/mL) and streptomycin (100 mg/mL). Preferably, the CD34 $^+$  cells are cultured in the presence of the cytokines for from 8 to 12 days.

[0014] Dendritic cells that form in the culture are isolated by panning as described above, with the exception that anti-CD1a and/or anti-CD14 antibodies are employed (both antibodies being commercially available, e.g. from Becton-Dickinson).

[0015] The MLR method comprises the following steps:

(1) providing a sample of responder cells; (2) providing a sample of inactivated stimulator cells such that the stimulator cells are allogeneic with respect to the responder cells and such that the stimulator cells consist of dendritic cells produced by the process comprising the steps of (a) treating CD34 $^+$  hematopoietic cells with TNF- $\alpha$  and IL-3 or with GM-CSF, and (b) isolating treated CD34 $^+$  hematopoietic cells that express the CD1a antigen; (3) co-culturing the responder cells and the inactivated stimulator cells; and (4) measuring a response of the responder cells.

[0016] Preferably, the sample of responder cells consists of CD4 $^+$  T cells from the peripheral blood of a patient who is to be the recipient of a transplant. Obtaining T cell populations employs techniques well known in the art which are fully described by DiSabato et al., eds., in *Meth. in Enzymol.*, Vol. 108 (1984). For example, the CD4 $^+$  T cells can be isolated as follows: first mononuclear cells are isolated from the peripheral blood and depleted of adherent cells; CD4 $^+$  T cells are then purified by depleting other cell types, for example by immunomagnetic depletion (e.g. with Dynabeads, Dynal, Oslo, Norway), or the like, using a cocktail of commercially available monoclonal antibodies, e.g. anti-CD14, anti-CD16, anti-CD20, anti-CD8, anti-CD40 (available from Becton-Dickinson and/or Ortho Diagnostic Systems, New Jersey). CD4 $^+$  populations having higher than 95% purity are typically achieved after two rounds of immunomagnetic depletion.

[0017] Stimulator cells used in the procedures described herein are dendritic cells derived from CD34 $^+$  hematopoietic progenitors cells obtained from a different person from that from whom the responder cells are taken; that is, the stimulator cells are allogeneic with respect to the responder cells. These cells are obtained as described above.

[0018] The stimulator cells are inactivated so that they can still carry out their stimulatory function but are inhibited from any other function that could obscure the response measured from the responder cells. Thus, the nature of the inactivation depends somewhat on the "read-out" of the assay. Preferably, the read-out, or response measured in the responder cells, is cellular proliferation. Other read-outs could also include such phenomena as cytokine production, cytolytic ability, and the like. Preferably, the stimulator cells are treated so that they are incapable of replication, but their antigen-processing machinery remains functional. This is conveniently accomplished by irradiating the cells, e.g. with about 1500 to 5000 R (gamma or X-radiation), preferably 3000 to 4000 R, before mixing with the responder cells.

[0019] Preferably, proliferation of the responder cells is determined by the uptake of tritiated thymidine using standard protocols. For example, from  $10$  to  $2.5 \times 10^4$  stimulator cells are added to  $2.4 \times 10^4$  allogeneic CD4 $^+$  T cells in 96-well round-bottom tissue-culture plates and are incubated for 4 days in the medium described above. After incubation, the cells are pulsed with 1  $\mu\text{Ci}$  of tritiated thymidine for 6 hours, and then they are harvested and measured for tritiated thymidine uptake, e.g. by scintillation counting.

## EXPERIMENTAL

[0020] The following examples serve to illustrate the present invention. Cell lines, reagents and their concentrations, temperatures, and the values of other variables are only to exemplify the application of the present invention and are not to be considered limitations thereof.

### Example 1

#### Generation of Human Dendritic Cells

[0021] After 12 days of culture in GM-CSF and TNF- $\alpha$ , cells generated from CD34 $^+$  cord blood hematopoietic pre-

cursor cells were processed for two-color fluorescence measurement. Briefly, cells were sequentially incubated with unconjugated monoclonal antibodies, phycoerythrin-conjugated (PE-conjugated) anti-mouse immunoglobulin, normal mouse serum, and monoclonal antibodies OKT6 (anti-CD1a from Ortho) or Leu-M3 (anti-CD14 from Becton-Dickinson), the monoclonal antibodies being directly labelled with fluorescein isothiocyanate (FITC). As shown in Figure 1, culturing CD34<sup>+</sup> cells for 12 days in the presence of GM-CSF and TNF- $\alpha$  allows the appearance of CD14<sup>+</sup> monocytic cells co-expressing the CD1a antigen. In addition, a population of CD14<sup>+</sup>CD1a<sup>+</sup> cells was consistently observed which represented 30-90% of the total CD1a population (range from 5 experiments). Within the monocytic lineage, expression of CD1a antigen is restricted to Langerhans cells. Figure 1A shows two-color fluorescence intensity from IgG<sub>1</sub>-FITC isotype control versus IgG<sub>2a</sub>-PE isotype control. Figure 1B shows two-color fluorescence intensity from OKT6-FITC (proportional to CD1a expression) versus Leu-M3-PE (proportional to CD14 expression). As shown in Figure 2, CD1a<sup>+</sup> cells were not detected at the onset of the culture and the CD1a antigen could first be observed after 4 days of culture in the presence of TNF- $\alpha$ , and expression increased until day 20. Whereas less than 5% of CD1a<sup>+</sup> cells were observed in the presence of IL-3, 5-15% CD1a<sup>+</sup> cells were detected in GM-CSF. A large proportion of CD1a<sup>+</sup> cells were detected in IL-3 and TNF- $\alpha$  (10-25%) and mainly in GM-CSF and TNF- $\alpha$  (20-60%) (range of 5 experiments after 12 days of culture). In terms of growth efficiency, IL-3 plus TNF- $\alpha$  is 2-3 times more potent than IL-3 alone, and GM-CSF plus TNF- $\alpha$  is 3-4 times more potent than GM-CSF alone (Figure 2). Thus, starting from 10<sup>5</sup> CD34<sup>+</sup> cells, after 12 days in culture, GM-CSF allowed the generation of 1-3 x 10<sup>6</sup> CD1a<sup>+</sup> cells, whereas in IL-3 plus TNF- $\alpha$  and GM-CSF plus TNF- $\alpha$  cultures of 2-3 x 10<sup>6</sup> cells were recovered. As GM-CSF plus TNF- $\alpha$  appeared to be the most potent combination of factors for generating CD1a<sup>+</sup> cells, all the cultures for the characterization of those cells contained GM-CSF plus TNF- $\alpha$ : Figure 2 shows the expansion of 10<sup>5</sup> CD34<sup>+</sup> cells (i) in the presence of IL-3 (solid triangles), (ii) IL-3 and TNF- $\alpha$  (open triangles), (iii) GM-CSF (solid squares), and (iv) GM-CSF and TNF- $\alpha$  (open squares). Solid lines indicate total cell numbers in the three experiments, and dashed lines indicated expression of CD1a antigen. Figures 2A and 2B represent data from two separate sets of experiments.

[0022] The morphology of the CD1a<sup>+</sup> cells generated by the method of the invention was studied using both light and electron microscopy. Adherent cells observed in GM-CSF alone display the classical aspect of regularly shaped macrophages, whereas those obtained in the presence of GM-CSF plus TNF- $\alpha$  have a typical aspect of dendritic cells with highly ramified dendrites, lobulated nucleus, and a villous surface with dendritic projections. Some of the CD1a<sup>+</sup> cells (about 1 in 5) possess organelles with double membrane joining, recalling the structure of Birbeck granules.

[0023] The phenotype of CD1a<sup>+</sup> cells generated after 12 days of culture in the presence of GM-CSF and TNF- $\alpha$  was determined by two-color fluorescence analysis. Depending on the experiment, the percentage of CD1a<sup>+</sup> cells co-expressing CD14 varied between 10 and 70%. The results presented in Table I below were obtained from experiments in which less than 15% of CD1a<sup>+</sup> cells co-expressed CD14; thus, both CD1a<sup>+</sup> cells and CD1a<sup>+</sup>CD14<sup>+</sup> cells were characterized. CD1a<sup>+</sup> cells co-expressed CD1c, CD4, and CD40, but did not express CD1b. In contrast, CD1a<sup>+</sup>CD14<sup>+</sup> cells did not express CD1c and only weakly expressed CD4 and CD40. Both CD1a<sup>+</sup> and CD14<sup>+</sup> cells were found to bear Fc $\gamma$ RII (CD32), Fc $\gamma$ RIII (CD16), and CR3 (CD11b), whereas only CD1a<sup>+</sup>CD14<sup>+</sup> cells expressed Fc $\gamma$ RI (CD64) and CR1 (CD35). CD1a<sup>+</sup> and CD1a<sup>+</sup>CD14<sup>+</sup> cells expressed both LFA1 $\alpha$  (CD11a) and LFA1 $\beta$  (CD18), but CD1a<sup>+</sup> cells exhibited higher levels of ICAM1 (CD54) than CD1a<sup>+</sup>CD14<sup>+</sup> cells. CD1a<sup>+</sup> cells expressed very high levels of HLA-DR (5-10 times more than CD14<sup>+</sup> cells). In contrast, CD1a<sup>+</sup>CD14<sup>+</sup> cells expressed high levels of HLA-DQ<sup>+</sup>.

Table I

Phenotype of Dendritic Cells Produced According to the Invention				
Antigen	Antibody	Reactivity with CD1a cells	Reactivity with CD14 cells	Source of Antibody (*)
CD1a	OKT6, IOT6a, T6, DMC1, L544	++	-	Ort, Hy8, Imu, Cou,
CD1b	IOT6b	-	-	Imu
CD1c	IOT6c	+	-	Imu
CD14	Leu-M3	+/-	+++	BD
CD4	IOT4	++	+/-	Imu
CD40	Mab 89	++	+/-	EP
CD16	Leu11b	+	+	BD
CD32	2E1	+/-	+/-	Imu
CD64	197	-	+	Med
CD21	CR2	-	-	BD
CD11b	IOM1	++	++	Imu
CD35	IOT17	-	++	Imu
CD54	84H10	+++	+	Imu
CD11a	SPVL7	++	++	Hy2
CD18	BL5	++	++	Imu
HLA-DR	L243	++++	++	BD
HLA-DQ	SPVL3	+++	+	Imu

\*Antibodies were obtained from sources as follows: Ort = Ortho Diagnostic Systems; Imu = Immunotech; Cou = Coulter (Hialeah, FL); BD = Becton-Dickinson; Med = Medarex Inc. (Lebanon, NH); Hy2 = Hybridoma, Vol. 2, pg. 423 (1983); Hy8 = Hybridoma, Vol. 8, pg. 199 (1989); EP=Eur. Pat. Appl. 89 403 491.7

## Example 2

### Proliferation of CD4<sup>+</sup> T Cells after Stimulation by Dendritic Cells

[0024] CD34<sup>+</sup> cells were cultured for 12 hours in accordance with the invention, after which they were irradiated with 4000 Rads to form stimulator cells for the experiment. From 10 to  $(2.4 \times 10^4)$  stimulator cells were seeded for  $2.5 \times 10^4$  resting CD4<sup>+</sup> T cells or other responder cells in round-bottomed microtest tissue culture plates in medium supplemented with 10% human AB<sup>+</sup> serum, as described below.

[0025] Dendritic cells were assayed for their capacity to induce resting allogeneic CD4<sup>+</sup> T cells (as responder cells) to proliferate. As shown in Figure 3A, cells cultured in the presence of IL-3 alone (solid triangles) induced marginal allogeneic CD4<sup>+</sup> T cell proliferation. In contrast, cells cultured in the presence of GM-CSF alone (solid squares), in the presence of IL-3 plus TNF- $\alpha$  (open triangles), and in the presence of GM-CSF plus TNF- $\alpha$  (open squares), induced a strong proliferation of allogeneic CD4<sup>+</sup> T cells. Depending on culture conditions of the CD34<sup>+</sup> progenitor cells, the optimal proliferation of the CD34<sup>+</sup> T cells was observed for different values of the ratio (stimulator cells)/(allogeneic CD34<sup>+</sup> T cells). In comparison with control values (CD34<sup>+</sup> T cells without stimulator cells), a 50-fold enhancement of tritiated thymidine uptake by CD4<sup>+</sup> T cells was observed at a ratio of 1:3.8 (range 1:3 to 1:25), 1:12.5 (range 1:10 to 1:35), and 1:360 (range 1:100 to 1:400) for cells cultured in the presence of GM-CSF alone, IL-3 plus TNF- $\alpha$ , and GM-CSF plus TNF- $\alpha$  respectively (ranges from 5 experiments).

[0026] In the Experiment illustrated in Figure 3B, the CD34<sup>+</sup> cells were cultured in the presence of GM-CSF and TNF- $\alpha$  for all experiments and the responder cells were adult peripheral blood (open squares), cord blood (open and solid triangles), and syngeneic cord blood CD4<sup>+</sup> T cells (solid squares). Figure 3B shows that allogeneic CD4<sup>+</sup> T cells

derived either from cord blood or from adult peripheral blood were equally stimulated, and that syngeneic CD4<sup>+</sup> T cells were stimulated to a much lower extent (response 20-fold weaker than allogeneic cells).

[0027] When, after 12 days of culture in the presence of GM-CSF and TNF- $\alpha$ , CD1a<sup>+</sup> cells were removed by immunomagnetic depletion (Figure 4), a strong loss of induction capacity was observed. A 50-fold enhancement of CD4<sup>+</sup> T cell proliferation was observed for a ratio (stimulator cells)/(allogeneic CD4<sup>+</sup> T cells) of 1:200 (range of 3 experiments 1:200 to 1:400) and 1:8 (range of 3 experiments 1:8 to 1:40) before and after CD1a<sup>+</sup> cells were depleted, respectively. After irradiation, the following were used as stimulator cells: total population (solid squares), population from which CD1a<sup>+</sup> cells had been removed by depletion (open squares), and control population from which depletion was carried out with an anti-IgG<sub>1</sub> isotype antibody (open triangles).

### Example 3

#### Generation of Cancer-specific CD8 Cytotoxic T Cells for Adoptive Immunotherapy

[0028] CD34<sup>+</sup> cells are isolated from the peripheral blood of a cancer patient and grown in the presence of GM-CSF and TNF- $\alpha$ , as described above. Peripheral blood CD8 cells are cryopreserved. Once dendritic cells are generated, CD8 T cells are thawed and mixed with the patient's cancer cells. After sensitization, CD8 T cells are expanded in the presence of IL-2, e.g. as described by Rosenberg, U.S. patent 4,690,915. Total cells are reinfused into the patient provided that they display tumor-specific cytotoxic activity. The tumor cells may be replaced by specific tumor antigens, e.g. van der Bruggen et al., *Science*, Vol. 254, pp. 1643 (1991).

#### Dendritic cells generated in the presence of GM-CSF and TNF- $\alpha$ are strong stimulators of resting allogeneic CD8<sup>+</sup> T cell proliferation

[0029] Dendritic cells generated from CD34<sup>+</sup> cord blood progenitors were cultured for 12 days and irradiated (4000 Rads), and then were used as stimulator cells for resting CD4<sup>+</sup> and CD8<sup>+</sup> T cells. From 10 to 2.5 x 10<sup>3</sup> stimulator cells were seeded for 2 x 10<sup>4</sup> resting T cells, in round-bottomed microtest tissue-culture plates, in medium supplemented with 10% human AB<sup>+</sup> serum, with or without 20 U/ml IL-2. After 5 days' incubation, cells were pulsed with 1  $\mu$ Ci of <sup>3</sup>H-thymidine for 8 hours, harvested and counted. Tests were carried out in triplicate and results were expressed as mean counts per minute.

[0030] The addition of IL-2 to the medium in which the CD4<sup>+</sup> and CD8<sup>+</sup> T cells were cultured indeed stimulated the proliferation of the CD4<sup>+</sup> and CD8<sup>+</sup> T cells, as shown by their <sup>3</sup>H-thymidine uptake. As shown in Figure 5, CD8<sup>+</sup> cells cultured in the presence of medium alone (open squares) showed very little proliferation whereas CD8<sup>+</sup> cells cultured in the presence of IL-2 (solid squares) showed much more proliferation; moreover, CD4<sup>+</sup> cells cultured in the presence of medium alone (open circles) showed little proliferation whereas CD4<sup>+</sup> cells cultured in the presence of IL-2 (solid circles) showed considerably more proliferation.

### Example 4

#### Generation of Primary and Secondary Antibody Responses in SCID-hu Mice

[0031] SCID-hu mice have allowed secondary responses to recall antigens but have not permitted the establishment of primary responses: e.g. Moller, *The SCID-hu Mouse*, Vol. 124 (Munksgaard, Copenhagen, 1991); Duchosal et al., *Nature*, Vol. 355, pp. 258 (1992); and Mosier et al., *Curr. Top. Microbiol. Immunol.*, Vol. 152, pp. 195 (1989). It is believed that this is due to a lack of reconstitution of the dendritic cell pool. As antigen-pulsed dendritic cells can efficiently induce an antibody response *in vivo*, e.g. Sornasse et al., *J. Exp. Med.*, Vol. 175, pp. 15 (1992), SCID-hu mice are reconstituted with the dendritic cells generated *in vitro* from the CD34 cells of the donor of human cells or from another donor sharing a compatible MHC. Dendritic cells are pulsed with the appropriate antigen prior to injection. Once the mouse displays antibody to the antigen, B cells are isolated from blood and other reconstituted organs and immortalized, e.g. by culturing in the CD40 system in the presence of Epstein-Barr virus as taught by Banchereau et al., *Science*, Vol. 251, pp. 70 (1991).

### Example 5

#### Treatment of AIDS by Adoptive Immunotherapy with Dendritic Cells Generated *In Vitro*

[0032] Dendritic cells are infected by HIV *in vitro* and HIV is found budding from the surface of the cells after 3-5 days in culture with HIV: e.g. Patterson, *J. Gen. Virol.*, Vol. 68, pgs. 1177-1181 (1987); Macatonia et al., *Immunology*,



Vol. 71, pgs. 38-45 (1990); and Knight et al., *Immunol. Lett.*, Vol. 19, pgs. 177-182 (1988). Evidence for *in vivo* infection of dendritic cells by HIV comes from the description of infection of Langerhans cells of the skin and reduction in the amount of MHC class II molecules in cells of the skin: e.g. Tschachler et al., *J. Invest. Dermatol.*, Vol. 88, pgs. 233-237 (1987); and Belsito et al., *N. Engl. J. Med.*, Vol. 310, pgs. 1279-1282 (1984). Furthermore, 3-21% of blood dendritic cells from HIV patients contain HIV, with a level of infection two orders of magnitude greater than that seen in other cell types: Macatonia et al., *Immunology*, Vol. 71, pgs. 38-45 (1990). The infection of dendritic cells by the virus blocks their ability to present antigen to T cells, and thus inhibits the recruitment/proliferation of the T cells which is necessary for a successful immune response.

[0033] CD34<sup>+</sup> cells of HIV patients are used to generate dendritic cells in accordance with the invention. The dendritic cells are then incubated with selected antigens and reinfused into the HIV patient.

## Claims

1. A process for the *in vitro* generation of human dendritic cells comprising the steps of:

- (a) culturing human CD34<sup>+</sup> hematopoietic cells (i) with GM-CSF, (ii) with TNF- $\alpha$  and IL-3, or (iii) with GM-CSF and TNF- $\alpha$ , thereby inducing the formation of CD1a<sup>+</sup> human dendritic cells from said CD34<sup>+</sup> hematopoietic cells; and
- (b) recovering said CD1a<sup>+</sup> human dendritic cells from said culture.

2. The process of claim 1 wherein the CD34<sup>+</sup> cells are cultured in the presence of GM-CSF.

3. The process of claim 1 wherein the CD34<sup>+</sup> cells are cultured in the presence of GM-CSF and TNF- $\alpha$ .

4. The process of claim 1 wherein the CD34<sup>+</sup> cells are cultured in the presence of TNF- $\alpha$  and IL-3.

5. A process according to any preceding claim wherein the recovered dendritic cells are pulsed with antigen.

6. A process according to any preceding claim wherein the CD34<sup>+</sup> cells are obtained from an HIV patient.

## Patentansprüche

1. Verfahren zur *in-vitro*-Erzeugung von humanen dendritischen Zellen, das die folgenden Schritte umfaßt:

- (a) Kultivieren humaner CD34-positiver hämatopoetischer Zellen (i) mit GM-CSF, (ii) mit TNF- $\alpha$  und IL-3 oder (iii) mit GM-CSF und TNF- $\alpha$ , wodurch die Bildung von CD1a-positiven humanen dendritischen Zellen aus den CD34-positiven hämatopoetischen Zellen induziert wird; und
- (b) Isolieren der CD1a-positiven humanen dendritischen Zellen aus der Kultur.

2. Verfahren gemäß Anspruch 1, wobei die CD34-positiven Zellen in Gegenwart von GM-CSF kultiviert werden.

3. Verfahren gemäß Anspruch 1, wobei die CD34-positiven Zellen in Gegenwart von GM-CSF und TNF- $\alpha$  kultiviert werden.

4. Verfahren gemäß Anspruch 1, wobei die CD34-positiven Zellen in Gegenwart von TNF- $\alpha$  und IL-3 kultiviert werden.

5. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die isolierten dendritischen Zellen mit Antigen gepulst werden.

6. Verfahren gemäß einem der vorstehenden Ansprüche, wobei die CD34-positiven Zellen von einem HIV-Patienten erhalten werden.

## Revendications

1. Procédé de production *in vitro* de cellules dendritiques humaines, comprenant les étapes consistant :

## EP 0 633 930 B1

(a) à cultiver des cellules hématopoïétiques CD34+ humaines (i) avec du GM-CSF, (ii) avec du TNF- $\alpha$  et de l'IL-3, ou (iii) avec du GM-CSF et du TNF- $\alpha$ , de façon à provoquer la formation de cellules dendritiques humaines CD1a+ à partir desdites cellules hématopoïétiques CD34+ ; et

(b) à récupérer de ladite culture lesdites cellules dendritiques humaines CD1a+.

5

2. Procédé selon la revendication 1, dans lequel les cellules CD34+ sont cultivées en présence de GM-CSF.

3. Procédé selon la revendication 1, dans lequel les cellules CD34+ sont cultivées en présence de GM-CSF et de TNF- $\alpha$ .

10

4. Procédé selon la revendication 1, dans lequel les cellules CD34+ sont cultivées en présence de TN- $\alpha$  et d'IL-3.

5. Procédé selon l'une quelconque des revendications précédentes, dans lequel les cellules dendritiques récupérées sont pulsées avec un antigène.

15

6. Procédé selon l'une quelconque des revendications précédentes, dans lequel les cellules CD34+ sont obtenues d'un patient à VIH.

20

25

30

35

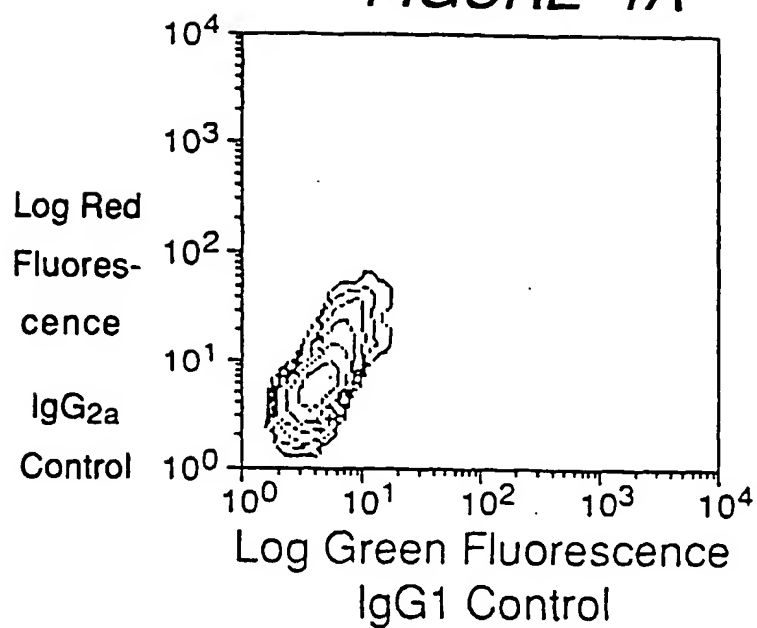
40

45

50

55

**FIGURE 1A**



**FIGURE 1B**

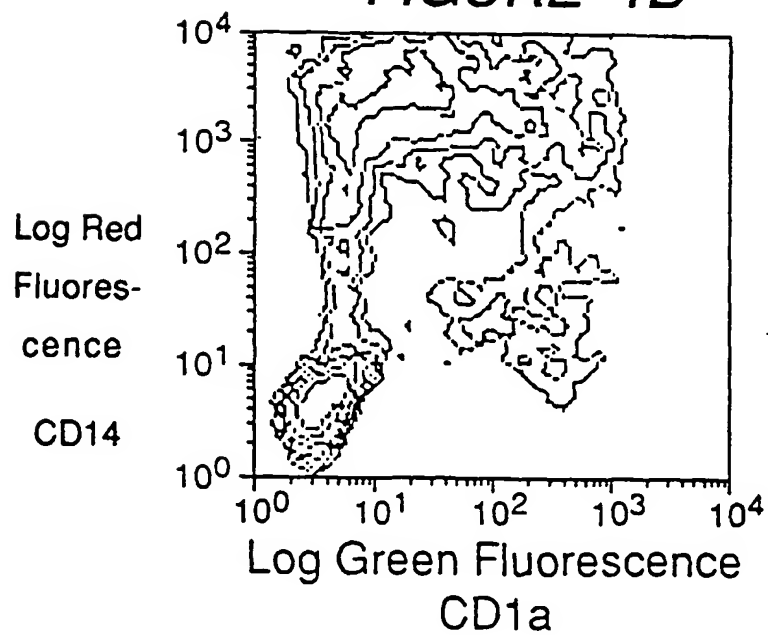
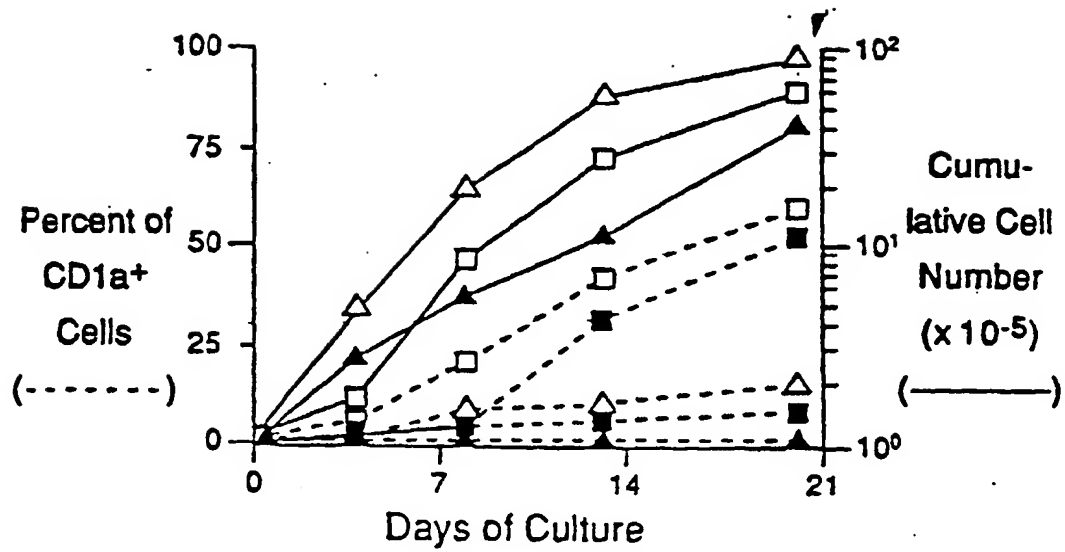
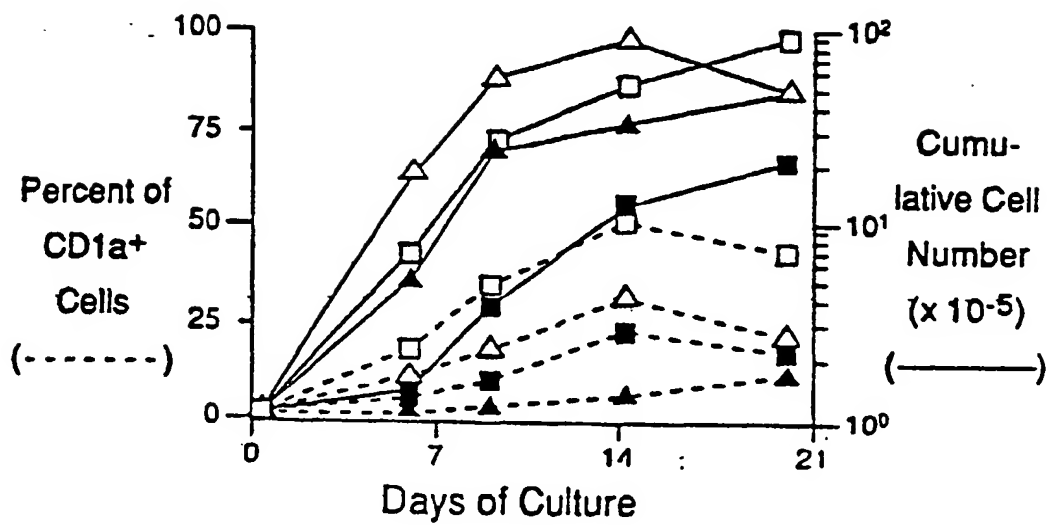


FIGURE 2A



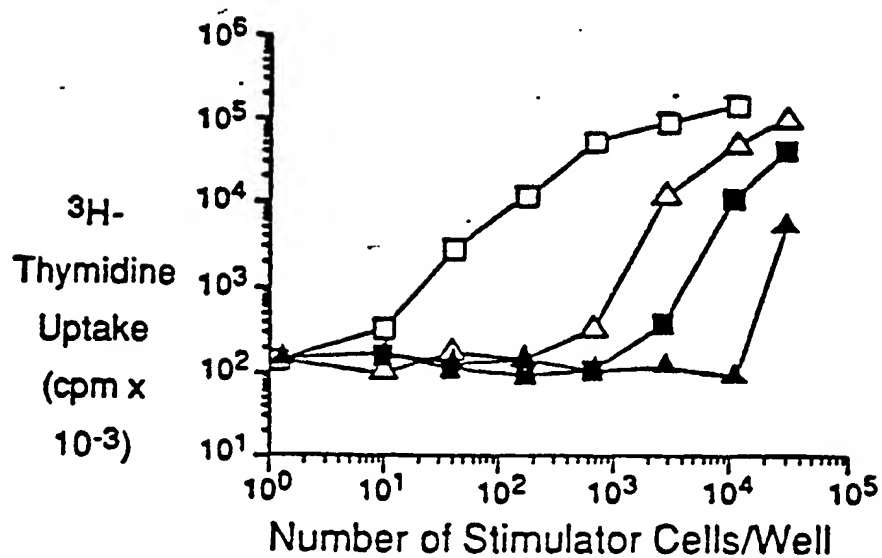
- △— IL-3 + TNFα
- ▲— IL-3
- GM-CSF + TNFα
- GM-CSF

FIGURE 2B



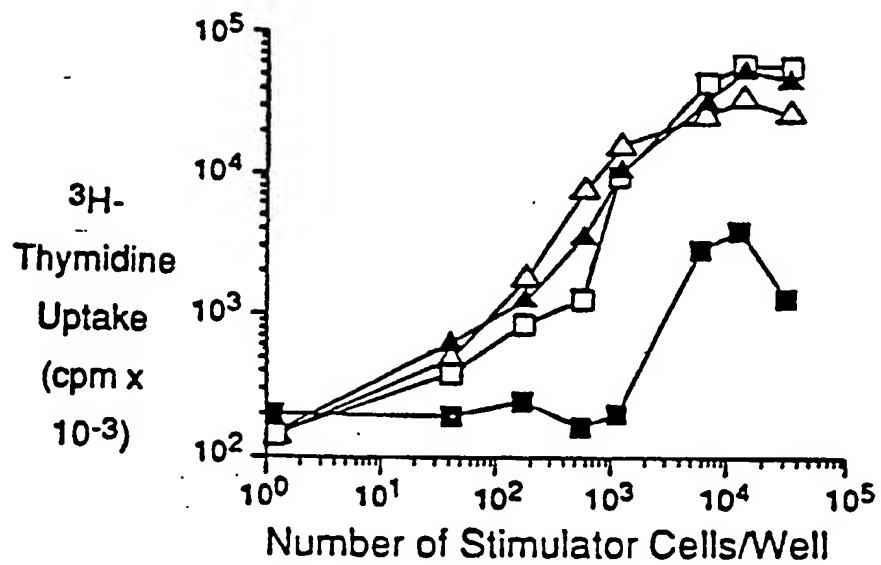
- △— IL-3 + TNF $\alpha$
- ▲— IL-3
- GM-CSF + TNF $\alpha$
- GM-CSF

FIGURE 3A



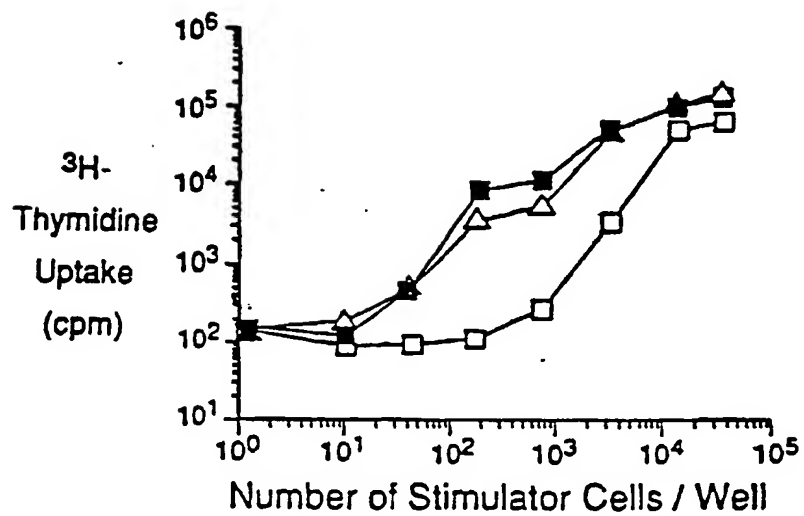
- GM-CSF + TNF $\alpha$
- GM-CSF
- △— IL-3 + TNF $\alpha$
- ▲— IL-3

FIGURE 3B



- Adult peripheral blood
- Syngeneic cord blood  $\text{CD4}^+$  T cells
- △— Cord blood
- ▲— Cord blood

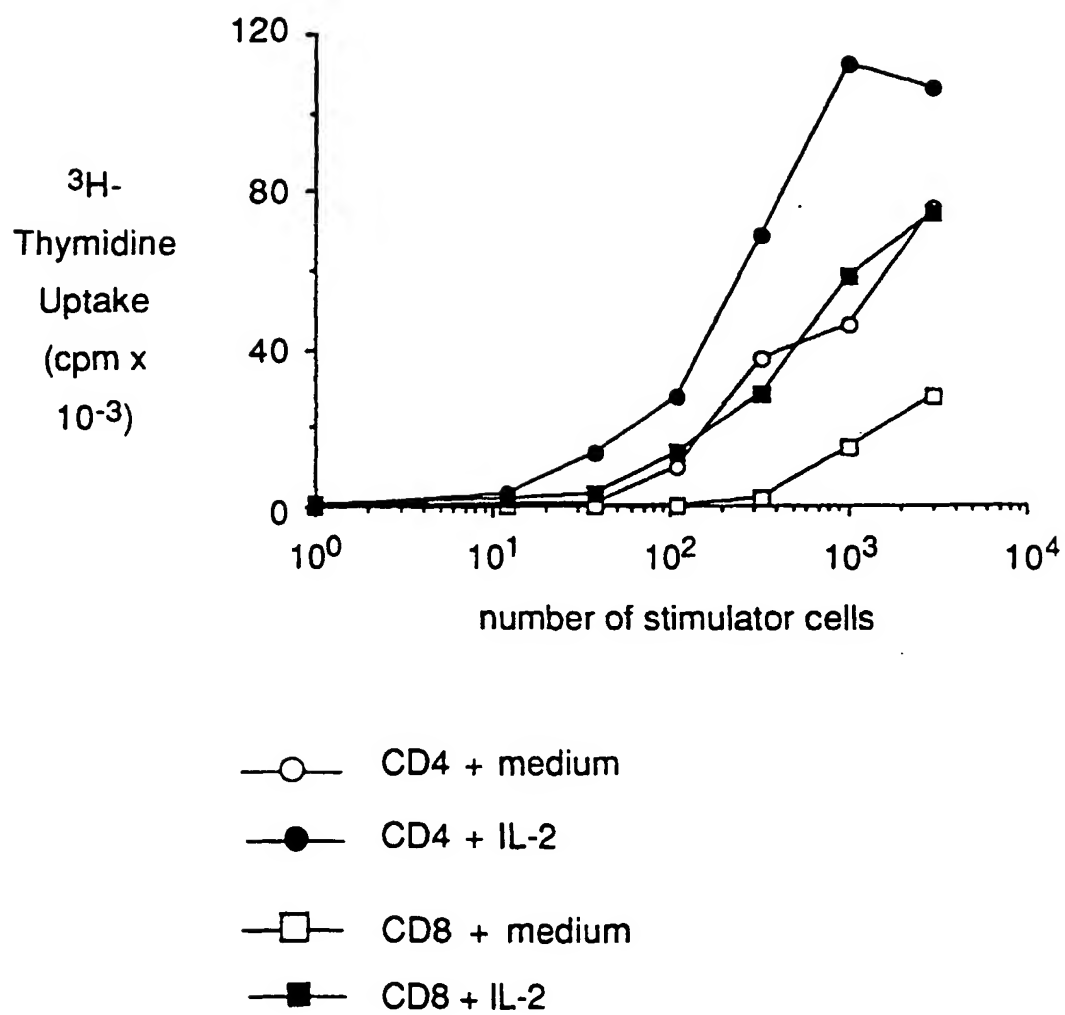
FIGURE 4



—■— total

—□— total minus CD1a<sup>+</sup> (immunomagnetic depletion)—△— total minus CD1a<sup>+</sup> (depleted with antibody)



*FIGURE 5*

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**